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RADIOCHEMICAL INVESTIGATION OF THE BEHAVIOR OF INORGANIC IONS UNDER SEPHADEX-GEL FILTRATION*

Application to the Microchemical Determination of PBI (Protein-bound Iodine) in Blood Serum

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With the utilization of Sephadex** first suggested by Porath and Flodin [1] in 1959, for gel filtration, an important step forward was realized in the removal of salts from protein solutions as well as generally in the fractioning of substances on the basis of their molecular magnitude. According to the most recent investigations of Porath [2, 3], the technique is successful, for instance, in providing a simple manner of separating proteins, peptides and aminoacids.

The gel filtration indicated is a column method in which the stationary phase is formed by a dextrane gel (Sephadex). This hydrophil substance is a polysaccharide for which the starting basis is dextrane. Its polymerization is induced so that the polysaccaride chains form a macromolecular, three-dimensional lattice of high stability. The polar character is determined by a high content of hydroxyl groups. Sephadex is practically insoluble in water and salt solutions but possesses high swelling characteristics. The substance is stable against weak acids and alkaline solutions. In practical operation, the swelled Sephadex is placed in columns, then filled with the experimental solution for separation of the components of the latter. Elution is then performed with the same solution utilized for swelling. Large-molecular substances do not enter into the gel phase but move along with the solution between the gel grains. They leave the column immediately after the passage of that amount of the solvent which corresponds to the dead

* Dedicated to Professor Doctor-Engineer Georg Gorbach on his 60th birthday.

** Pharmacia, Uppsala, Sweden.

volume of the column. The latter signifies that amount of liquid which is contained between the gel grains of the Sephadex column. Substances of smaller molecular magnitude and/or ions penetrate the gel grains and are eluted more slowly due to their longer path.

The investigations carried out so far [1-4] were effected with macro-columns which made it possible to separate milligram amounts of substances of different molecular magnitude. The subject of this communication is radiochemical investigations on the behavior of milligram and/or submicrogram amounts of inorganic ions in Sephadex micro-columns. The results obtained led to the application of this technique for the separation of protein-bound iodine (PBI) from inorganic iodine in blood serum (normal rate 0-2 µg iodide per 100 ml of blood serum) which is essential in diagnosis of thyroid function.

1. Behavior of Inorganic Ions under Sephadex-Gel Filtration

Material

The filtration experiments were carried out with Sephadex G-25 which has the following properties: absorption of water, 2.5 g/g of dry matter; density when moist, 1.009; grain dimension when dry, 50-100 mesh.

In the water content of a Sephadex column, we can distinguish two kinds of watery phases: the water in the gel grains (internal volume = V_i) and the water surrounding the gel grains (dead volume = V_o). V_i is calculated from the dry weight of the amount of Sephadex utilized and its capacity for absorption of water. Part of the internal water volume is firmly bonded as hydration water and separates out during transport of the solvent. The total volume of a Sephadex column therefore amounts to $V_{total} = V_o + V_i + V_g$ in which V_g signifies the dry volume of the Sephadex utilized.

For complete exclusion from the gel phase, the molecular weights and primarily the molecular magnitude are decisive. With Sephadex G-25, particles with a molecular weight of 2,000-3,000 are still being excluded.

We used reagents of the Merck Company, and for adequate measurement of activity, strongly diluted solutions of the following radioactive source (factory-prepared solutions from Amersham, England): $Na^{131}I$, $Na^{35}SO_4$, $^{65}ZnCl_2$, $^{60}CoCl_2$.

Methodology

Sephadex was allowed to swell for two hours either with distilled water or different concentrations of sodium chloride solutions. In order

to obtain constant velocity of throughflow, fine gel particles must be eliminated by flotation before filling the columns. These fine particles clog the columns under continued use and reduce the velocity of throughflow. When filling the columns, the formation of air bubbles should be prevented. For each experimental series, we always used the same column with an interior diameter of 10 mm. The bottom of the column is formed by a fused screen which is covered by a filter-paper disc to prevent the passage of gel grains. The columns are filled with swelled Sephadex to a height of 120 mm. Another filter-paper disc was placed on the filter mass to prevent turbulence in the latter when introducing the experimental solutions. After preparation, these columns were left to stand for at least two days under running water which allows the gel grains to settle and pack so that optimum and constant characteristics of separation are obtained. The experimental solution was then poured into the column at a given volume and permitted to be completely absorbed. Subsequently some of the elution liquid was poured into the column to prevent the entry of air. The column was then closed by a rubber plug which was pierced by a capillary tube. The latter is immersed in a container with elution liquid. In order to obtain a constant velocity of throughflow of 1 ml/80 sec, the point of the capillary tube was provided with the suitable diameter. Beginning with the admission of the experimental solution, the eluate was collected in amounts of 1 ml. The radioactivity of the individual amounts was measured on a bore-hole scintillation counter FH 421 (γ-measuring head Z 11) in connection with the radiation-measuring instrument FH 49 (Frieseke & Hoepfner Company).

Sephadex columns treated with this method retain constant characteristics of separation without any change in the velocity of throughflow and the filling of the column for several months. The amounts of the different ions and substances admitted can be determined in each case reproducibly in the eluate.

Findings

The investigations described below under behavior of inorganic ions in Sephadex columns prepared in different ways were carried out for the purpose of being able to utilize this technique of separation for quantitative separation of serum-protein iodine from inorganic iodine (in amounts from submicrogram to tenths of 1 milligram).

a) $^{131}I^-$, $^{35}SO_4^{2-}$, $^{60}Co^{2+}$ and $^{65}Zn^{2+}$ and different carriers in Sephadex swelled with distilled water:

As described above, we prepared a column of Sephadex swelled in distilled water. The volume of the experimental solution introduced was always 0.5 ml. We determined radioactivity for individual amounts of 1 milliliter of the eluate.

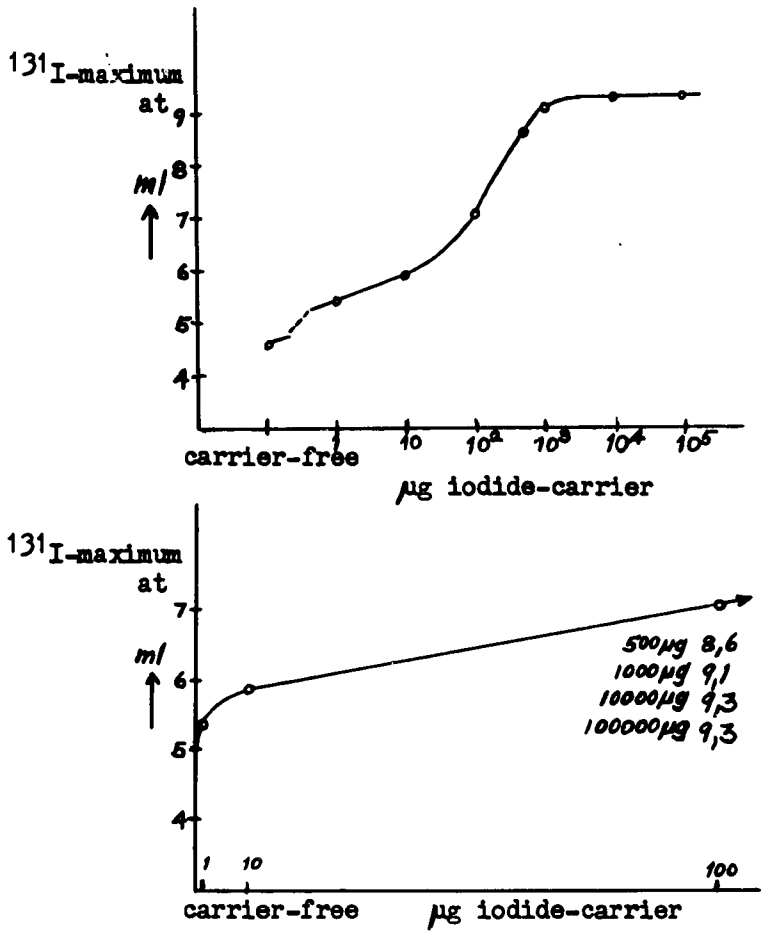

Figure 1. Displacement of iodide maximum in eluate volume in dependence on carrier amount.

Iodide: Iodide introduced without carrier (order of magnitude: nanogram) occurs in the eluate immediately after the dead volume (3 ml) has passed through. The maximum of radio-iodide lies at 4.6 ml and the last still observed radio-iodide in the seventh milliliter. When increasing the carrier amount, the maximum of activity and consequently the yield of iodide is displaced in the direction of a larger elution volume and finally appears, after 1,000 µg of iodide carrier, always at the same point in the eluate. Figure 1 shows the position of the maxima of activity as indicator for the principal amount of iodide during elution in dependence on the given carrier amount (represented linearly as well as half-logarithmically).

Sulphate: Sulphate shows the same behavior under the same experimental conditions. Radio-sulphate separated from the carrier appears in the eluate again immediately after passage of the dead volume. The addition of 10 mg of inactive carrier produces the same displacement backward of the maxima of activity as for iodide.

Cobalt and Zinc: Diluted factory-prepared solutions of $^{65}Zn^{2+}$ and $^{60}Co^{2+}$ react essentially different from the anions investigated. The cations contained in 0.5 ml of test solution introduced become fixed in the top section of the column and can no longer be eluted by intensive washing with distilled water (in amounts up to 100 ml). It is possible to dislocate cations so retained by other cations. Sephadex swelled in distilled water was saturated with carrier-free ^{60}Co-solution, filtered and washed four times with distilled water. The initial activity of 30,300 Imp/min was reduced by this only by about 3,500. With 0.9%-salt solution, the entire activity could be immediately eluted. Sodium therefore dislocated cobalt in the sense of an ion-exchange absorption.

b) Carrier-free $^{131}I^-$ and $^{35}SO_4^{2-}$ in sodium-chloride solution of increasing concentration as elution liquid:

For more detailed investigation of the behavior of $^{131}I^-$ and $^{35}SO_4^{2-}$ in prepared Sephadex columns, we then determined the position of their activity maxima at concentration changes of the sodium-chloride solutions used as elution medium.

Carrier-free iodine reacted under distilled water as described in a), i.e. the activity maximum occurred in the fourth milliliter of the eluate (Figure 2). Before any change in sodium-chloride concentration, the respective solution was allowed to flow for six hours through the same column. When using an 0.001% sodium-chloride solution, the maximum corresponding to ^{131}I becomes displaced into the fifth milliliter of the eluate. Further increases of concentration of the salt solution produce a gradual increase of the volume which is required for elution of activity.

As will be seen further from Figure 2, the total of carrier-free radio-iodide is found in the fourth to sixth milliliter of eluate when utilizing distilled water. Increase of the sodium-chloride concentration enlarges the basis of the activity curves with simultaneous decrease of the absolute heights of the activity maxima occurring. However, in all experiments, the total volume of the eluted $^{131}I^-$ was equal to the volume introduced and corresponded to 42,000 Imp/min. A similar enlargement of the basis of the activity curves could be noted in the experiments described under a). As control, the column containing 10%-salt solution finally had all salt removed with distilled water.

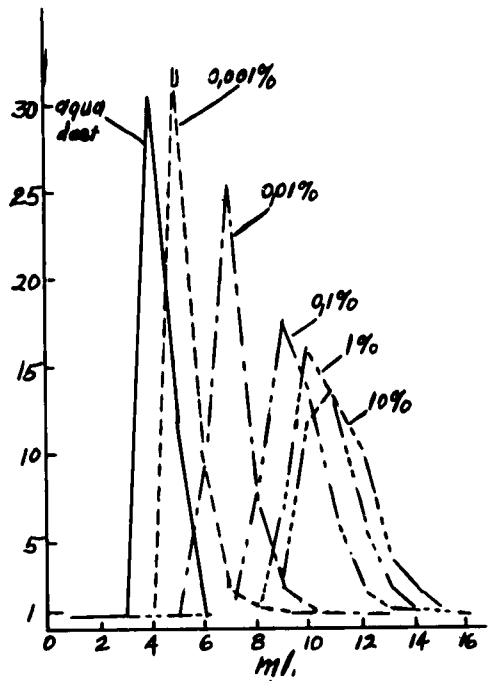

Figure 2. Distribution of carrier-free $^{131}I^-$ in eluate at changes of concentration of sodium-chloride solution.

Carrier-free iodide then introduced reacted completely identical as at the beginning to the experimental series.

We obtained the same results in analogous experiments with carrier-free $^{35}SO_4^{2-}$. Here activity was measured with the β-counter FHZ 11. If we then selected, for the results just mentioned, the same graphic representation as in Figure 1., we then obtain essentially the same curves.

c) $^{131}I^-$, $^{35}SO_4^{2-}$, $^{60}Co^{2+}$ and $^{65}Zn^{2+}$ and different carriers in Sephadex swelled with 0.9%-salt solution:

For the separation of serum proteins and inorganic ions, an 0.9% sodium-chloride solution is suitable as swelling and elution liquid. We therefore investigated the behavior of the above substances in such columns and with different carrier volumes.

According to the findings of these experiments, the ions of these substances penetrate the gel grains and are therefore all eluted more slowly, as a function of the increased path they are required to travel, independently of the volume of the carrier added. They are found in the eluate beginning with about the eighth to the thirteenth milliliter. The

carrier volume utilized in these experiments varied from 0 to 10 mg. Even a carrier volume of 100 mg resulted in no displacement and/or no essential enlargement of the basis of the activity curves.

2. Application of gel filtration to microchemical determination of PBI in blood serum

In differential diagnosis of thyroid disorders, microchemical determination of iodine bonded to serum protein (PBI) offers valuable help. The method suggested by Spitzy et al [5] has proved its worth by application to different clinical and experimental problematics [6-18] since its publication. The separation of PBI from inorganic iodine takes place through precipitation with trichloro-acetic acid with subsequent washing of the precipitated protein in the incineration retort without transfer of the sample. In normal cases (0 to 2 µg%), washing twice is sufficient for complete removal of the inorganic serum iodine. However, if inorganic iodine or inorganic iodine compounds were given for therapy, then an inorganic iodine level of, for instance, 300 µg% required washing ten times in order to obtain non-altered PBI values. Even the difficult determination of complete elution of the protein precipitate from inorganic iodine through negative result of the catalytic iodine reaction in the washing liquid does not exclude a share of inorganic iodine entrained into the protein precipitate, and consequently artificially increased PBI values. On the other hand, repeated washing of the protein precipitate means, in addition to a considerable expenditure of time, the risk of a protein loss. Nor are these difficulties eliminated by the use of other deproteinization media, e.g. zinc sulphate and sodium hydroxide; on the contrary, Skanse and Hedenskog [19] observed quantitative protein precipitation in this case only after three hours at rest. Vilkki [20] suggests, for effective separation from

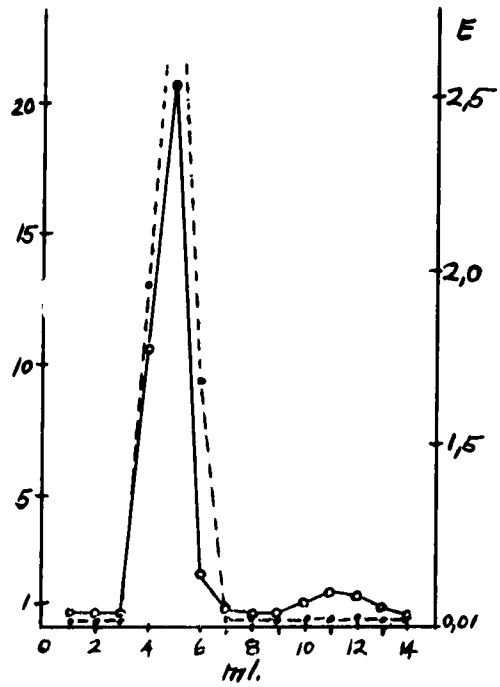

Figure 3. Distribution of activity and protein of blood serum biologically labelled with $^{131}I^-$ after separation through Sephadex G-25 columns. Abscissae: ml of eluate. Ordinata: activity and/or extinction at 280 nm.
——o—— = activity.
-·-·-●-·-·- = protein.

inorganic iodine, dialysis by running water but this requires in itself 12 hours for each determination. Moreover, this method requires the transfer of the specimens into different receptacles and incurrs the risk of clogging the dialysis membrane.

By utilizing gel filtration with Sephadex, these difficulties are completely obviated. The method permits rapid quantitative separation of PBI, even when there is a high excess of inorganic iodine (up to 20,000 µg%).

Preliminary Experimentation

Blood serum was first biologically labelled with $^{131}I^-$. Carrier-free $^{131}I^-$ was injected into laboratory animals and the serum obtained 48 hours later. The latter contains $^{131}I^-$ to about 90% in protein-bound form.

Sephadex G-25 was left to swell in 0.9% sodium-chloride solution and filled into columns in the manner described above. The velocity of throughflow again amounted to 1 ml/80 sec. At velocities over 1 ml/min, less satisfactory separation was obtained. We introduced 0.5 ml, in each case, of the blood serum labelled with $^{131}I^-$ into the column and eluted with 0.9% sodium-chloride solution. We then collected amounts of 1 ml and measured their activity. Simultaneously we determined the distribution of the serum protein in the eluate through measurement at 280 mm with the Zeiss spectrophotometer PMQ-II.

Figure 3 shows the separation of the blood serum in regard to activity and protein which agreed in numerous tests. Up to a dead volume of 3 ml, neither radio-iodine nor protein show themselves. Up to the sixth milliliter, we then obtain radio-iodine quantitatively bonded to the serum protein. The next three milliliters contain neither radio-iodine nor protein and inorganic radio-iodine becomes eluted beginning with about the tenth to the thirteenth milliliter.

The separation of PBI from inorganic iodine in the serum is consequently sharp and entirely adequate. An attempt was therefore made to transpose this separation technique to the microchemical determination of PBI content of inactive serum specimens.

Methodology and Findings

We examined in each case 0.5 ml of different blood-serum specimens. The first three ml of the eluate were discarded and the following three milliliters were transferred directly into the incineration retort of the apparatus for iodine determination. The serum-protein samples so obtained were subjected directly to incineration, by-passing trichloro-acetic acid

precipitation, and microchemical determination of iodine according to the technique described in [5]. The table below contains a comparison of the findings of PBI determination after trichloro-acetic acid precipitation and after separation by Sephadex filtration.

Table 1

Serum No.	µg-% PBI after precipitation with trichloro-acetic acid	µg-% PBI after separation with Sephadex
1	9.9	10.2
2	2.3	2.2
3	2.3	2.3
4	3.0	3.0
5	2.1	1.9
6	4.0	4.2
7	3.1	2.9
8	4.0	3.9
9	2.5	2.4
10	12.8	12.6
11	7.7	7.5
12	5.9	6.1

The numerical blank-test value was the same for both methods with 0.009 µg of iodine and both methods have the same accuracy.

The advantage of the new method becomes especially obvious from the following results:

0.5-ml samples of a blood serum for which PBI content was determined as 3.5 µg-% both by trichloro-acetic acid precipitation and by Sephadex separation, had 100 µg of iodide added in the form of sodium iodide. This corresponds to an inorganic iodide content of 20,000 µg-%. The separation of inorganic iodine through Sephadex columns was quantitative and PBI determination gave 3.6 µg-% in three parallel tests. Samples of the same serum precipitated with trichloro-acetic acid and eluted twice produced, however, 188 µg-% after PBI determination.

Discussion

Molecular screening through Sephadex-gel filtration, according to the present findings and amounts of substance, can be utilized for amounts in magnitudes of micro- and submicrograms. It produces quantitative results and these are reproducible in regard to the location of the substances in the eluate.

The findings reported under 1. show that inorganic ions react differently to Sephadex in dependence on their concentration and the selection of the swelling and of the elution liquid. Anions in solutions of low ion strength are repelled by Sephadex but cations become fixed immediately upon contact with the gel. When using 0.9% salt solution, anions and cations appear quantitatively at the same point in the eluate independently of their concentration.

Apparently, the molecular screening is superposed by charge effects in experimental solutions of low ion strength. Sephadex gel probably contains small amounts of carboxyl groups which are difficult to demonstrate with customary methods of analysis. At extremely low ion strengths, we may therefore expect ion-exchange absorption which explains our findings with $^{60}Co^{2+}$. On the other hand, the carboxyl groups in the gel structure repel the anions as is shown by our experimental findings with $^{131}I^-$ and $^{35}SO_4^{2-}$ *.

The utilization of gel filtration with Sephadex to microchemical PBI determination in blood sera eliminates the many difficulties which result from the presence of too much inorganic iodine in the blood. The usual content of inorganic serum iodide generally amounts to only 0.0 to 2.0 µg-% but may increase appreciably under exogenous iodine introduction. Since only 0.5 ml of blood serum are needed for microchemical iodine determination, 0.00 to 0.01 µg iodide must be separated from the protein-bound iodine. High amounts of iodine introduced for therapy or absorbed from ambient surroundings increase, in extreme cases, the serum content of inorganic iodine to about 20,000 µg-%. In such cases, accurate results in PBI determination (normal content 3.0 to 7.6 µg-%) could previously be obtained through protein precipitation only with difficulties and at a considerable expenditure of time and effort.

The findings reported under 2. confirm the satisfactory applicability of Sephadex separation both for infinitely small as well as for extremely high amounts of iodide. Separation is sharp, quantitative, and reproducible. Properly prepared Sephadex columns retain the same characteristics of separation for months. However, in comparison to trichloro-acetic acid precipitation and/or dialysis, an appreciable advantage is produced in regard to working time. Separation of PBI is terminated within nine minutes and, after eleven more minutes for throughflow

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* We are indebted to Dr. J. Porath, Institute for Biochemistry, Upsala University, for a valuable written discussion of our findings. According to his communication, he utilized the ion-exclusion of positively charged Sephadex derivatives for the separation of basic polypeptides and obtained effects which were similar to our findings with iodide ions.

of 0.9% sodium-chloride solution, the column is again operative. At the present time, we are experimenting an automatic apparatus on which we will report later.

Gel filtration with Sephadex, according to preliminary tests of thyroxine labelled with $^{131}I^-$, is also highly suitable for investigations of the bonding capability of proteins to various substances.

Summary

In comparison with dialysis and the methods of protein precipitation, gel filtration offers considerable advantages in the removal of salts from protein solutions. The behavior of inorganic ions (milligram to submicrogram amounts) was studied on Sephadex micro-columns with the aid of radioactive isotopes. Inorganic ions were found to behave diversely depending on the concentration chosen and the swelling and elution liquid. In 0.9% sodium-chloride solution, submicrogram amounts of cations and anions also enter the gel phase; they can be separated quantitatively and reproducibly from the protein which remains excluded from the gel phase. On the basis of the experimental data furnished, the gel filtration with Sephadex G-25 can be used for the separation of protein-bound serum iodine (PBI) from inorganic iodine. In contrast to the previous methods of separation, it is likewise useful for blood serums with extremely augmented iodide content. The saving in time and work is a further advantage.

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